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Research on ancient DNA in the Near East

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Abstract: *In the early 1990s, when studies of ancient DNA became possible, new perspectives of analyzing archaeological data also developed. Nowadays, because the methodology related to ancient DNA research is well developed, it has been used to reveal several aspects of human history and interaction. Here we review the basic concepts, methodologies, and recent developments in the field of ancient DNA studies with a special reference to the Near East. This includes not only human but also animal and bacterial DNA.*

Key words: archaeogenetics, aDNA, mtDNA, tuberculosis, animal domestication

Introduction

Human genomes accumulate mutations gradually over time. The forces of genetic drift and natural selection either cause these changes to disappear or to become established in the population. By the end of the 1990s, Amorim (1999) introduced the term "archaeogenetics" in reference to using information regarding genetic differences between humans to understand demographic events that took place in the past.

Pioneering studies of human genetic diversity date back to 1970s when Cavalli-Sforza published a report on the diversity of European populations based on classic protein markers (see Cavalli-Sforza et al. 1994 for a review). In the mid-eighties, great opportunities for studying human diversity arose with the invention of polymerase chain reaction (PCR). This method allowed for the amplification of desired DNA fragments to millions of copies, thus further analyses, such as sequencing, were possible (Mullis et al. 1986). In the early 1990s researchers focused on mitochondrial DNA (mtDNA). The human mitochondrial genome is a small circular DNA molecule (16569 base pairs, bp) (Anderson et al. 1981). Due to a lack of DNA repair systems in mitochondrion, mutations in mtDNA occur ten times more often than in nuclear DNA (Brown et al. 1979). MtDNA is maternally inherited as a single unit. This feature makes mtDNA a marker of choice for studying human phylogeny. The first mtDNA population data was published by Torroni et al. (1993). They studied single nucleotide polymorphisms (SNPs) determined by restriction fragment length polymorphism (RFLP) patterns in more than 300 Amerindian mtDNA segments and showed that they form four distinct phylogenetic groups. These four groups were referred to as haplogroups and named A, B, C, and D (Torroni et al. 1993). A haplogroup is defined as a group of haplotypes sharing common ancestry, whilst a haplotype is a variant of a DNA sequence characterized by a unique set of nucleotide substitutions at polymorphic sites. There are two fragments of

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mtDNA that are usually analyzed: HVRI and HVRII (Hyper Variable Region I and II). These regions are located in the displacement loop (D-loop)—a noncoding segment of mtDNA (Anderson et al. 1981). Over time the accessibility of DNA sequencing has become broader and analyses of whole mtDNA genomes are available (Torroni et al. 2006).

Today, a great number of mtDNA segments have been sequenced allowing for the construction of a worldwide human mtDNA phylogeny (the most recent phylogenetic tree built on the basis of the whole mitochondrial genome was published by van Oven & Kayser 2008).

Other than mtDNA analyses, markers in nuclear DNA are also widely used for phylogenetic studies. Analyses of Y chromosome markers have become particularly frequent in the last few years (Jobling & Tyler-Smith 1995, 2003). The Y chromosome contains the largest non-recombining portion of the whole human genome, and because of this, is considered one of the most informative systems. Because Y chromosomes are inherited via the paternal line, genetic data obtained from the Y chromosome reflects aspects of population histories different than those obtained from mtDNA analyses. Due to a smaller migration rate of males compared to females, specific Y chromosome lineages are even more geographically clustered than those of mtDNA (Seielstad et al. 1998). Identification of vast numbers of informative SNPs (e.g., Underhill et al. 1997, 2000) allow for the construction of Y chromosome phylogenetic trees analogical to those based on mtDNA (Karafet et al. 2008).

Since the mid-1980s, theories constructed on the basis of the archaeogenetic approach could be compared with data obtained directly from archaeological samples. The term generally used to refer to DNA extracted from such material is "ancient DNA" (aDNA).

In the 1980s the first report documenting the successful isolation of aDNA was published (Higuchi et al. 1984) and the isolation of DNA from human bone became possible (Hagelberg et al. 1989). Since then, the field of aDNA became important in revealing details of human history. Except for minor differences, the analysis of aDNA is carried out using similar methods to those used in studying population and forensic genetics of contemporary populations.

Studies of aDNA are burdened by several constraints, most of which result from the poor post-mortem preservation of the DNA molecule. Following an organism's death its DNA begins to decay. The action of endogenous and exogenous nucleases (DNA-cutting enzymes) leads to rapid DNA cleavage. Physical and chemical factors also affect DNA. Oxidation leads to the modification of nitrogenous bases and of the structure's sugar backbone. Hydrolysis leads to deamination and strand breakage (Lindahl 1993). The chemical and physical modifications of DNA cause problems with its amplification and sequencing. Oxidation products such as hydantoins (Hoss et al. 1996) and DNA-protein cross-links (Poinar et al. 1998) inhibit PCR. The hydrolytic deamination of cytosine to thymine nucleotides leads to incorrect sequence determination (Gilbert et al. 2003a, 2003b). Therefore, only small fragments of DNA, up to 300 bp long, are usually detected in archaeological samples (Pääbo 1989; Pääbo et al. 1989; Handt et al. 1996; Hoss et al. 1996). The degradation of DNA to small fragments occurs relatively quickly following death and does not proceed further on. The experiments carried out by Pääbo reveal that molecules of the same length, of approximately 300 bp, were present in samples removed from archaeological bone dated between 4000 and 13,000 years ago (Pääbo et al. 1989).

It is typically considered that DNA degradation depends mostly on environmental conditions of the deposition environment while time is not a critical factor. It is estimated that DNA molecules will not survive longer than 10,000 years in temperate climates and not more

than 100,000 to 500,000 years under specific conditions such as rapid desiccation, low temperatures, or anaerobic environments (Willerslev & Cooper 2005).

The most important problem in all aDNA studies is the contamination of samples with exogenous DNA. Extremely sensitive methods allowing for amplification of minute amounts of DNA from ancient specimens make these studies prone to contamination with modern DNA. When even a single DNA molecule can be amplified to millions of copies, one unguarded moment can lead to amplification of modern DNA instead of aDNA. A strict protocol was proposed to minimize the risk of contamination (Cooper & Poinar 2001; Poinar 2003; Willerslev & Cooper 2005) (**Table 1**).

Table 1. Contamination controls for ancient DNA studies.

Sample collection and handling	When possible samples dedicated for aDNA study should be handled with gloves from the moment of excavation. This task is usually hard to achieve as most of the material for DNA analysis comes from museum collections, but it gives strong support to the authenticity of the results.
Physically isolated laboratory area for aDNA isolation and PCR set-up	In such a laboratory no experiments involving modern DNA are allowed. All the surfaces should be UV radiated and cleaned frequently with bleach. Staff members should wear protective clothing, face masks, and gloves. Only sterile disposables are allowed and special laboratory equipment is used e.g., filtered pipette tips and laminar flow cabinets etc. Recently, systems retaining positive pressure in the laboratory have been installed.
Reaction blanks	For each DNA isolation and PCR there must be negative controls performed to track down reagent contamination.
Reproducibility	Identical results should be obtained for at least two DNA extractions and PCRs from each sample.
Independent replication	At least the key results should be replicated in another laboratory to avoid intra-laboratory contaminations.
Cloning of PCR products	Sequences obtained by direct PCR sequencing should be confirmed by cloning and the sequencing of several clones. This allows for the tracking of incorrect sequence determination caused by chemical modification of the aDNA.
Quantification	The number of DNA molecules in aDNA extracts should be assessed via Real-Time or competitive PCR at least for a subset of samples. When there are low amounts (<1000 copies) of DNA in the extract, an exclusion of incorrectly determined sequence may be impossible.
Biochemical preservation	The possibility of aDNA preservation may be assessed by measuring a level of protein diagenesis in the samples. It is usually carried out by measuring amino acid racemisation or collagen content.
Associated remains	When possible, an analysis of aDNA from non-human species from the same archaeological site should be used as a comparison for sample DNA survival.

The guidelines presented in **Table 1**, especially independent DNA amplification in a second laboratory, are often difficult to achieve. It is important that any precautions undertaken should be described in detail for any study. For example, it is typical for researchers to present clone sequences, the results of amino acid racemisation analysis, mtDNA profiles of the laboratory staff, and the quantification results; if not in the main text, at least in supplementary materials.

Unfortunately, even strict adherence to these guidelines does not provide a hundred percent guarantee of authenticity, but results obtained in compliance with these rules are widely accepted among researchers. Studying DNA from archaeological samples is a costly and time consuming process. However, the results of these studies are most often worth every effort.

Genetic landscape of the Near East

Until recently researchers believed that the first migration of anatomically modern humans out of Africa occurred via the so called "Northern Route", along the Nile River, through Sinai and the Near East (for our purposes the "Near East" includes the Asian portion of Anatolia, Syria, Lebanon, Israel, Egypt, Iran, Iraq, and the Arab Peninsula). Somewhere in this region this first out-of-Africa human population split and colonized Europe and Southeast Asia. New data from the analysis of the entire mitochondrial genome suggests that the initial dispersal from Africa occurred via the "Southern Route". These new clues come from the analysis of "relict" populations from the Malay Peninsula (Orang Asli) (Macaulay et al. 2005) and Andaman Island (Thangaraj et al. 2005). According to these authors, the first modern humans crossed the Bab-el-Mandeb strait and proceeded along the coast of the Indian Ocean and finally settled Australia. The majority of analyzed samples from both locations belonged to the superhaplogroups named M and N. These branches of the mtDNA phylogeny are dated to ~63,000 years ago, just after the first migration out of Africa. The specific mtDNA lineages identified in these studies, namely M21, M22, M31, and M32, are derived directly from the ancestral M lineage ~60,000 years ago. This information suggests that the initial peopling of Southeast Asia took place instantly after populations left Africa. Initial population growth in western Southeast Asia is dated to 52,000 years ago, earlier than in North and Central Asia (49,000 years ago) and Europe and the Near East (42,000 and 40,000 years ago, respectively) (Atkinson et al. 2008). In light of this, it seems that the peopling of the Near East and Europe was just an offshoot of the initial migration along the Southeast Asian coast (Macaulay et al. 2005). Such a scenario was recently supported by the analysis of archaeological material. Mellars (2006a) demonstrated similarity in lithic assemblages and other findings between African sites such as "Howieson's Poort" levels at Klasies River, "Still Bay" levels at Blombos Cave, and Enkapune y Muto (dated to 75,000 to 40,000 BP) and Southeastern Asian sites such as Patne in western India and Batadomba Lena in Sri Lanka (dated between 34,000 and 30,000 BP). The expansion of modern humans from Africa is dated on the basis of mtDNA to having occurred around 60,000 years ago, but there are earlier human remains known from outside of Africa. Skeletons from Skhul and Qafzeh, Israel are dated to ~90,000 years according to Mellars (2006a, 2006b). However, these could reflect earlier, unsuccessful dispersals.

The modern genetic diversity of the Near East is the result of thousands of years of population movements and demographic change. Numerous studies of European mtDNA lineages also include samples from the Near East (Di Rienzo & Wilson 1991). The most complete

studies take advantage of nearly 1300 HVRII sequences from all of the Near East including Egypt and Sudan (Richards et al. 2000). Haplogroups present in the Near Eastern gene pool are derivatives of superhaplogrups such as M, N, and R which arrived with the initial out-of-Africa migration. The most frequent ones are HV (30%), H (25%), U (22%), J (10%), and T (9%); additionally, less frequent clades were represented including: K, U1, U2, U3, U4, U5, U7, N1b, I, W, and X.

Surprising findings came from the examination of the Druze population from Galilee, Israel. The Druze is a population united by religion, existing today in Syria, Lebanon, Israel, and Jordan. Recent examination of their mtDNA revealed a relatively high frequency of the haplogroup X (13.1%) (Shlush et al. 2008). Unlike other mtDNA haplogroups, X does not exibit a geographic pattern. Its major subhaplogroup X1 is widely dispersed throughout North Africa whereas X2 is widespread from Europe to Northern America but in very low frequencies (Reidla et al. 2003). Druze is the first population identified where both subhaplogroups are represented, and their diversity is high (Shlush et al. 2008). A proposed explanation for these results is that Druze of Galilee represent the "refugium" population where haplogroup X remained since ancient times (Shlush et al. 2008) when it was more frequent. There are several other factors that could produce such a genetic structure in the Druze population, among them the sampling pattern, a recent bottleneck, founder effect, or migrations from other populations. All of these were eliminated in this study: the sampling process was designed to minimize typing of maternally related individuals. A bottleneck or the founder effect at the time when the population was established is unlikely because these processes would result in low genetic diversity within the population. Populations with the smallest genetic distances to the Druze are the Turks, Armenians, Iranians, and Egyptians. Simulations of different patterns of nonrandom migrations from these populations to the Druze show that it is unlikely that these could have shaped the current Druze genetic structure (Shlush et al. 2008).

Knowledge of Y chromosome variability in the Near East region is fragmentary. Similarly to mtDNA, Y chromosome haplogroups are shared among European populations. Most prevalent are E3b, J, J2, I, L, N, K2, and R1 (Nebel et al. 2001; Al-Zahery et al. 2003).

Recently, a Y chromosome study was carried for the Lebanese population. Nearly a thousand Lebanese men were sampled and typed for fifty-eight Y chromosome SNPs. Samples came from residents from five geographic regions belonging to different religions namely: Muslims, Christians, and the Druze. The purpose of this investigation was to study Y-chromosomal variation and to determine differences between groups based on either geographic or religious membership, and also to discover whether recent historic events such as the Muslim expansion from the beginning of the 7th century CE, the crusades from the 11th-13th centuries CE, and the Ottoman expansion in the beginning of the 16th century CE, left a noticeable impact on the modern genetic structure of the Lebanese (Zalloua et al. 2008).

The largest proportion of variation among subpopulations was detected when religion was used as a subdividing factor. The Lebanese data set was categorized into 8 haplogroups (Eb3, G, I, J*(J2), J2, K2, L, and R1b). This allowed for comparison with data sets of western Europeans, Arabs from the Arabian Peninsula, and Turks—the assumed source populations for the historic migrations into Lebanon. Simulations carried out to test the probability that a haplogroup could reach its contemporary frequency by drift alone and with a possible historic migration influence allowed the authors to create a specific hypothesis: that a portion of haplogroup J*(J2) chromosomes were introduced to Lebanese Muslims during the Muslim expansion and that a part of I and R1b chromosomes was introduced to Lebanese Christians

via migrations from western Europe possibly during the crusades. This last finding was also supported by a Y chromosome STR (Short Tandem Repeats) analysis. Surprisingly, four centuries of Ottoman existence in the region had no visible impact on the genetic structure of Lebanon (Zalloua et al. 2008).

Other studies of genetic variation focused on Jews (Hammer et al. 2000; Nebel et al. 2000; Nebel et al. 2001; Behar et al. 2003; Nebel et al. 2005), Samaritans (Shen et al. 2004), Kurdish groups (Nasidze et al. 2005), and Iraqis (Al-Zahery et al. 2003).

A detailed characterization of the genetic diversity in the Near East is beyond the scope of this review, as the amount of data on modern populations would overwhelm the few results of aDNA studies. It is obvious however that a significant amount of contemporary data is available for comparison with and to authenticate archaeological data.

Human aDNA studies from Near Eastern archaeological sites

DNA preservation

There is ongoing debate concerning DNA studies in the Near East. Doubts concerning the authenticity of some of the results arose following the publication of the results of DNA found in papyri samples from Egypt by Marota et al. (2002). The authors were trying to amplify chloroplast DNA from modern, 10- and 100-year old papyri specimens from the Museo del Papiro di Siracusa, and from specimens from archaeological sites dating between 1500 BCE to 700 CE. Positive amplification results were obtained only for recent specimens up to 100 years old. Competitive PCR experiments allowed the authors to estimate the DNA copy number in each sample. Based on their results, there was a half-life of DNA in sampled papyri of 21.5 years. This was in agreement with a theoretical DNA half-life of 20.8 years at 35°C (assuming 35°C as an average annual temperature) (Marota et al. 2002). These results reveal that the complete decay of DNA in archaeological samples in hot environments will take no more than 800 years. The second line of evidence comes from amino acid racemisation analysis. Earlier experiments revealed that there is a strong correlation between DNA decay and the extent of aspartic acid racemisation (Poinar et al. 1996). Amino acids, the main component of proteins, normally occur as D-enantiomers. Following the death of an organism, part of them converts into L-enantiomers. This process depends on similar factors as those causing DNA decay: temperature, humidity, and pH. Studies carried out by Poinar et al. (1996) revealed that an aspartic acid D/L enantiomers ratio higher than 0.08 strongly suggests complete DNA decay. The asp D/L ratio in all papyri samples from the archaeological sites exceeded 0.08, confirming the absence of DNA (Marota et al. 2002).

These calculations were in agreement with the results of Krings et al. (1999), whose attempts to extract DNA from 132 mummies from Pre-Dynastic Egypt were positive in only two specimens.

The conclusions of Marota et al. (2002) and of Krings et al. (1999) were challenged by Zink and Nerlich (2003c) who carried out extensive studies of pathogen DNA detection in Egyptian samples. They argued that the temperature estimates of Marota et al. (2002) were too high and that average temperatures in Egyptian tombs do not exceed 15–25°C. The majority of samples that failed to yield reliable DNA sequences came from sites that suffered from annual Nile inundations (e.g., Krings et al. (1999) samples came from Minshat Abu

Omar in the Nile delta) and this was not the case of tombs e.g., in Thebes/West and Abydos from where most of the samples analyzed by the authors came from. They also pointed out that Egyptian mummies undergo rapid desiccation during the mummification process, particularly due to the use of natron (composition of sodium salts) as a drying agent. Rapid desiccation leads to good macroscopic preservation of archaeological remains and strongly favors DNA preservation.

Gilbert et al. (2005) criticized arguments posed by Zink and Nerlich (2003c) as being too general and imprecise. For example, natron was not used in the mummification process during Predynastic times, and burial locations such as the Valley of the Kings (suggested by Zink and Nerlich (2003c) as being extremely dry) have been subject to flooding in recent years.

This debate demonstrates that the isolation of DNA from samples buried in hot environments remains somewhat controversial. With respect to these doubts, one should be cautious when dealing with reports on genetic analyses from the Near East.

Population genetics based on aDNA studies

The first isolation of human DNA from an ancient specimen, which also happens to be linked to the Near East, occurred during the pre-PCR era when Pääbo (1985) cloned fragments of a DNA sequence from the brain tissue of an Egyptian mummy. However, the obtained sequence was most probably the result of contamination; regardless, this work paved the way for aDNA research.

There are few published studies of mtDNA from Near Eastern archaeological sites. Most of them were undertaken to understand ethnic relationships between contemporary and ancient populations or to study kinship structure at archaeological sites. In some cases the purpose was to genetically characterize individual cases. Unfortunately, published studies are often fragmentary and do not fulfill the accepted criteria of authentication of aDNA results (see above).

A short report on the analysis of human remains from Tell Kurdu, Turkey (6000–4500 BCE) was published by Mekel-Bobrov and Lahn (2004). The HVRI region of mtDNA was successfully amplified in 11 of 14 samples. Three distinct mtDNA haplotypes were found. Haplotype sharing was attributed to kinship between individuals (Mekel-Bobrov & Lahn 2004). Their study boasted a success rate of nearly 80%, which, because of the age of the material, cast doubts regarding the authenticity of the data. The presented report lacks information concerning the mtDNA profiles of the researchers, cloning of the obtained PCR products, and laboratory procedures.

Bones of individuals from Tell Halula, Syria were also analysed using mtDNA to explore kinship structure (Fernandez et al. 2008). The site was occupied between 7800 and 5500 BCE. The genetic relationships between the inhabitants discovered in several buildings were tested by means of mtDNA analysis. Specimens were selected from Pre-Pottery Neolithic B levels (8800 BP). DNA was successfully amplified in 20 of the 50 individuals. Relatedness was suggested between individuals sharing the same HVRI haplotype, but the report lacks additional statistical analyses. Unfortunately, as in many other studies, contamination precautions were poorly described. The authors mentioned cloning of PCR products, amino acid racemisation analysis, and quantification of DNA molecules in extracts via Real-Time PCR. However, the results of these analyses were not presented in the main text or in the supplementary material.

There was a successful attempt to recover DNA from a 5300 year-old specimen from Israel (Agamy 2002). Of the 14 specimens analysed from Wadi Maqoch, Jordan Valley, Israel, dated to the Chalcolithic period, only one yielded amplifiable DNA. The obtained sequence was characterized by two polymorphic sites and its haplotype was different from those of the investigators (i.e., contamination was not an issue). The results were not published except for in a conference abstract. Nevertheless, the greatest value of the study was to show that, despite unfavorable conditions for DNA preservation, it is possible (although somewhat inefficient) to obtain DNA from material as ancient as this in the Near East.

One of the most complete aDNA studies involved analyzing material from the Dakhleh Oasis, Egypt. This report includes a detailed description of the procedures undertaken to avoid contamination. All samples were collected immediately following excavation, and the field staff wore latex gloves. Bone preparation and DNA isolation were carried out in a Paleo-DNA laboratory especially designed for ancient DNA work (Graver et al. 2001).

The investigators focused on the Kellis 2 cemetery associated with the ancient town of Kellis. This necropolis was in use between 300 and 390 CE and included ~3000 burials. DNA isolation was conducted for 50 skeletal samples from the cemetery. The aim of the study was to characterize the ancient population from the Dakhleh Oasis. To allow for inferences regarding population changes at the oasis, 94 contemporary samples were also analyzed. Previous genetic studies of Egyptian, Nubian, and Sudanese populations allowed for distinguishing between two mtDNA types: the so called "southern" (Sub-Saharan) and "northern" (Eurasian) (for details see: Chen et al. 1995; Krings et al. 1999). To obtain the frequencies of these mtDNA types, amplification of the HVRI region and three RFLP markers was conducted. The authors succeeded in analysing RFLP markers in 34 samples and HVRI sequences in 18 of the samples. Both populations, ancient and contemporary, fit the north-south clinal distribution of "southern" and "northern" mtDNA types (Graver et al. 2001). However, significant differences were found between these populations. Based on an increased frequency of Hpa I 3592 (+) haplotypes in the contemporary Dakhlehian population, the authors suggested that, since Roman times, gene flow from the Sub-Saharan region has affected gene frequencies of individuals from the oasis.

In some cases the aim of the aDNA study was to determine the region of origin of a single individual. Such a task is extremely difficult to achieve because of the wide geographic distribution of mtDNA haplogroups. Moreover, constant human movement and the presence of identical HVRI haplotypes in different maternal lineages render genetic investigations very difficult. Nevertheless, recently such an attempt was undertaken. The genetic characterization concerned the skeleton attributed to the evangelist Luke. St. Luke was born in Antioch, Syria. Following his death he was buried in Thebes and was probably later transferred to Padua, Italy (Vernesi et al. 2001). A tooth sample from the body traditionally ascribed to St. Luke was obtained in 1998. Amplification of mtDNA was successful and yielded a sequence belonging to haplogroup pre-HV. This sequence was compared with a data set of 49 southern Syrians, 48 Greeks, and 96 Turks and to nearly 3000 Europeans (Simoni et al. 2000). Standard methods (N-J tree, haplotype network) failed to provide any clues on the possible origin of the DNA sequence obtained for the Padua skeleton. However, application of statistical methods used in epidemiology resulted in nearly a 3-fold higher probability of Syrian (Near East) origin for the considered sequence (Vernesi et al. 2001). This seems to be a limitation of genetic investigations when there are no maternal relatives for comparison as it was, for example, in the case

of the putative heart of Louis XVII of France (Jehaes et al. 1998, 2001) or in the case of the identification of the Romanov family (Gill et al. 1994).

All of the above cited studies are based on mtDNA analyses. Autosomal or Y- chromosomal data would allow for a more detailed analysis, especially when inferring on kinship structure. However, amplification of nuclear markers from ancient specimens is extremely difficult and there have only been a few successful attempts (Keyser-Tracqui et al. 2003; Haak et al. 2008).

Molecular sexing

Sex determination of archaeological specimens is frequently hampered by poor preservation and is especially difficult when dealing with infant or subadult remains. Genetic sex determination is routinely carried out in forensic cases. There are several PCR-based methods of molecular sexing. The most common one is based on the amplification of a fragment of the amelogenin gene. The amelogenin locus is located on both sex chromosomes but there is a 6 bp deletion on chromosome X, so amplification of this locus results in two PCR products of differing lengths, usually 106 and 112 bp for males (who have both X and Y alleles) and only one product (112 bp) for females (Sullivan et al. 1993). Frequently a fragment of the male specific Y chromosome SRY region is co-amplified with a fragment of amelogenin gene for an additional sex confirmation (Santos et al. 1998).

Amelogenin PCR-based sex determinations are also suitable for archaeological remains (Faerman et al. 1995; Stone et al. 1996). Interesting results were reported by Faerman et al. (1998) who studied infant remains from Askhelon, Israel. In the sewer beneath a 4th century CE Roman bathhouse, a deposit of nearly 100 newborn infant bones was discovered. Infanticide was an accepted practice during Roman times and it was typical for female offspring to be treated in such a way (Harris 1994). Genetic analyses were performed on 43 left femora excavated from the sewer. Of 19 successful DNA amplifications 14 were designated as male and 5 as female. This was in contrast to the expected higher frequency of females. One possible explanation suggests that this bathhouse was a brothel, and the infants deposited there were the offspring of courtesans. In this specific case girls would have been more valuable than boys (Faerman et al. 1995, 1998).

Disease

Analyses of aDNA in human remains are not solely restricted to human DNA. In the last 16 years several cases of DNA of ancient bacteria and other human pathogens were reported (see Drancourt & Raoult 2005 for a review). Methods of isolation of bacterial DNA from archaeological remains are similar to those used in the isolation of human DNA. However, more stringent isolation protocols are required when dealing with *Mycobacteria* or other bacteria with lipid-rich cell walls.

Generally there are two strategies for detecting bacterial or parasitic species in ancient specimens. Non-specific methods involve the amplification of DNA with PCR primers from a broad range of bacterial taxa, and specific species determinations are based on the sequencing of clones obtained from the PCR products. Second strategy grounds on amplification

with species-specific PCR primers, thus species identifications are based on the presence or absence of PCR products.

Similar contamination precautions, such as using a separate aDNA laboratory, PCR product cloning, and the determination of the number of DNA molecule copies in the tissue extracts are usually employed. Similar to human DNA studies, because of an abundance of microorganisms in the environment, it is difficult to distinguish between ancient and modern (contaminant) sequences (Rollo & Marota 1999). Additional palaeoecological criteria were proposed when dealing with the DNA of microorganisms. The ecophysiology and the distribution of species identified by DNA analysis should be checked for consistency with the environmental conditions of the archaeological site from which the studied remains derive (Rollo & Marota 1999).

The first successful identification of pathogen DNA in an archaeological sample was reported by Spigelman and Lemma (1993). This pioneering work was criticized for its methodology, because the methodological standards were still under development at this early stage in ancient DNA studies. Eventually their findings were revised and later confirmed (Spigelman et al. 2002). Despite criticism, the new field of aDNA studies emerged. Since then the analysis of ancient pathogen DNA has allowed for the identification of a wide spectrum of diseases in archaeological material such as: tuberculosis (Taylor et al. 1996), leprosy (Haas et al. 2000a), malaria (Zink et al. 2001a), and the plague (Drancourt et al. 1998). Most of the publications present case studies of single or a few individuals and only a few of them include larger population sets.

The majority of the data obtained for ancient pathogenic DNA is related to tuberculosis (TB). Studies of TB aDNA are facilitated by the preservation of mycobacterial DNA in archaeological samples due to the resistance of its cell walls to degradation (Rafi et al. 1994; Gernaey et al. 2001).

The disease is caused by a group of related bacterial species known as the *Mycobacterium tuberculosis* complex. Usually the bacteria attack lungs (pulmonary TB), but in less than 10% of cases bone is also affected. Bone changes include vertebral collapse (Pott's disease), periosteal lesions, and osteomyelitis (Ortner & Putschar 1981). Skeletal changes allow for an easy morphological identification and the selection of remains for DNA typing. Molecular confirmation of *Mycobacterium* DNA presence is usually based on PCR amplification of a short fragment of a IS6110 repetitive element (Spigelmann & Lemma 1993; Taylor et al. 1996; Donoghue et al. 1998; Haas et al. 2000b; Zink et al. 2001a; Spigelman et al. 2002), which is present in up to 20 copies in *Mycobacterium tuberculosis* complex genomes.

Several studies of TB DNA include samples from the Near East. In the classic study by Spigelman and Lemma (1993), TB was detected in the spine of a child from Byzantine Turkey. The oldest remains with genetically confirmed TB come from the archaeological site of Atlit-Yam. This site is located about 10 km south of Haifa, Israel. Radiocarbon dates correspond to the Pre-Pottery Neolithic C period. At present, the site is covered by the sea and is situated 300 m offshore (Hershkovitz & Galili 1990). Skeletons of women and infants from the site show morphological changes consistent with tuberculosis. For DNA isolation the ribs, arm bones (women), and long bones (infants) were chosen. In both samples PCR products for the IS6110 fragment were obtained. Additionally, the presence of *M. tuberculosis* was confirmed by the amplification of a specific region exhibiting the deletion defining strain TbD1, which is widespread in modern populations (Hershkovitz et al. 2008). The specific confirmation of *M. tuberculosis* bacilli and the absence of *M. bovis* in individuals from the early Neolithic site

is relevant evidence in light of ongoing discussions concerning the origins of *M. tuberculosis*. Recent phylogenetic analyses (e.g., Brosch et al. 2002; Mostowy et al. 2002) reveal that the postulated provenance of *M. tuberculosis* from *M. bovis* is not likely. Findings from Atlit-Yam support the theory that the two microorganisms evolved separately.

Not only bone fragments can be used in the amplification of *M. tuberculosis* complex DNA in archaeological samples. During excavations at the Byzantine basilica in the Negev Desert at Karkur (600 CE), a calcified pleura was found in one of the human remains. Pleural calcification is typical for the chronic form of TB. Genetic analysis of this specimen identified *M. tuberculosis* DNA (Donoghue et al. 1998).

The largest number of samples typed for TB was taken from an ancient Egyptian population from the Thebes West necropolis by Zink et al. (2001b, 2005). The selected tombs in this cemetery dated from the Middle Kingdom (2050–1650 BCE) to the Late Period (500 BCE). For the molecular analysis, more than 50 samples were selected, most of which exhibited visible skeletal lesions. However, there were also samples with nonspecific skeletal changes or without any macroscopic pathological conditions. Several samples from the necropolis at Abydos, dated to 3050–2650 BCE, were also included. In cases in which the amplification of human DNA (ß-actin and amelogenin) was positive (30 samples), the *M. tuberculosis* complex was identified in 9 of the samples (30%). In some situations the *M. tuberculosis* complex was detected in samples exhibiting no skeletal pathology for the condition. Recently, 50 additional samples from the same site were typed for TB and revealed similar infection frequencies (Zink et al. 2003b). Based on the relative frequent presence of *Mycobacterium* in remains bearing no sign of tuberculosis, the authors suggest that TB was widespread in ancient Egypt.

Apart from TB, other diseases have also been genetically confirmed by studying aDNA. Donoghue et al. (2005) focused on a co-infection of *M. tuberculosis* and *M. leprae*. Individuals from various locations exhibiting morphological changes associated with either multibacillary leprosy, tuberculosis, or both were tested. The set of samples included an individual buried in a tomb in Akeldama (Himmon Valley, Israel, 1st c. CE) and ten individuals from the Dakhleh Oasis, Egypt (4th c. CE). Detection of *M. leprae* DNA was based on the amplification of a species-specific repetitive DNA sequence called RLEP. 30% of samples, including the specimen from Akeldama and four specimens from Dakhleh, tested positive for both pathogens. These results contradict the theory of cross-immunity, according to which an infection with one of the pathogens renders an individual immune to another (Murhekar et al. 1995). The authors favor the interpretation that the decline of leprosy cases was not due to the postulated cross-immunity, but higher death-rates of co-infected patients (Donoghue et al. 2005).

A case of leprosy was also confirmed by DNA analysis in a skeleton from Bet Guvrin dated to 300–600 CE (Spigelman & Donoghue 2001). Non-specific pathological changes in the lower extremities were diagnosed as Madura's foot or leprosy. PCR amplification with two pairs of primers, specific for *M. leprae,* targeting the RLEP repetitive sequence and the 18-kD antigen was successful and provided a diagnosis of leprosy.

Several attempts were made to identify the DNA of *Plasmodium falciparum* in the human remains, one of the parasites responsible for malaria. Rabino Massa et al. (2000) used immunological tests to screen 80 mummies from the site of Gebelen near Luxor, Egypt dated to 3200 BCE. The *Plasmodium* antigen (histidine-rich protein PfHRP-2) was found in 43% of samples (and in 92% of samples with porotic hyperostosis). Such a high frequency of cases caused doubts concerning the specificity of the antigen based test (Nerlich et al. 2008). A positive *Plasmodium* identification via immunological methods was also reported for a Granville

mummy—a 50 year old woman from the site of Gurna, Egypt dated to 700 BCE (Miller et al. 1994). Reexamination of this specimen using PCR-based methods yielded negative results. These results could be due to the differential preservation of DNA and proteins in this individual, but serious doubt concerning the reliability of the immunological test arose (Taylor et al. 1997). A recent survey of Nerlich et al. (2008) yielded more realistic results. 91 specimens were screened for *Plasmodium* DNA, 7 from the Predynastic to Early Dynastic site of Abydos (3500–2800 BCE), 42 from a Middle Kingdom tomb in Thebes West (2050–1650 BCE), and 42 from other tombs also from Thebes West, dated from the Middle Kingdom until the Late Period. PCR of a fragment of a *pfcrt* gene (*P. falciparum* chloroquine-resistance transporter gene) was attempted and resulted in two positive amplifications. The specificity of the obtained PCR products was confirmed by carrying out the sequencing in two independent laboratories.

Zink et al. (2001c) screened 450 individuals from Thebes West searching for *Corynebacterium diphtheriae,* the pathogenic bacteria responsible for diphtheria. Of the 40 samples that yielded amplifiable DNA, one positive PCR result was obtained with starters targeting eubacterial 16S rDNA. The presence of *Corynebacterium* spp. DNA was confirmed in only one specimen, the head of a woman buried in Dra Abu el Nega (Thebes West) dated to 1580–1080 BCE. A specific identification of *Corynebacterium diphtheriae* species was not possible. However, in conjunction with inscriptions found in the tomb describing the treatment of a disease bearing resemblance to diphtheria, the presence of *C. diphtheriae* seems likely. The presence of *Corynebacterium diphtheriae* in the ancient mummy was not surprising, since diphtheria is common even in contemporary Egypt.

Another parasite detected in human remains via aDNA analysis is the *Leishmania donovani* complex; the parasite causing leishmaniasis. Zink et al. (2006) searched for *L. donovani* DNA in 91 bone samples from the above-mentioned Egyptian sites of Thebes West and Abydos and in 70 samples from Nubian sites at Kulubnarti, Sudan. These sites were early Christian cemeteries dated from 550 to 750 CE and from 750 to 1500 CE. DNA sequences specific to *Leishmania* spp. were PCR amplified from 4 Egyptian and 9 Nubian samples. Based on frequencies of bacterial presence, the authors conclude that leishmaniasis was endemic in Nubia during the 6th–8th centuries CE. An examination of earlier samples would most likely have led to similar results since Sudan (or East Africa in general) is considered as a place of origin of visceral leishmaniasis (Zink et al. 2006). As all the Egyptian samples containing *L. donovani* DNA came exclusively from a Middle Kingdom tomb and no samples from earlier periods yielded bacteria-positive results, the authors suggest that the introduction of leishmaniasis to Egypt may have taken place during the Middle Kingdom. The presence of *L. donovani* in Egypt implies close trade contacts between these countries as the distribution of *L. donovani* is closely associated with its vector the phlebotomine sandfly, which is absent in Nile Valley.

Zink et al. (2000) described a single case of bacteremia discovered when an infant mummy from the Thebes West cemetery was studied. The mummy was dated to 1000–750 BCE. Genetic analysis revealed the presence of the DNA of several bacterial species, including *Escherichia coli*, *Frateuria auranta*, and *Halobacillus* spp. Post mortem spread of *E. coli* through the body was ruled out. Isolation of these bacteria from a metatarsal led the authors to conclude that the child must have suffered from both bacteremia and septicemia.

DNA analysis can also be used to detect some genetic diseases. A specimen from an 8 year-old child tested positive for ß-thalassemia (Filon et al. 1995). The examined skull was found in an Ottoman grave, dated between the 16th and 19th centuries CE at Akhziv, on

the northern coast of Israel. The skull exhibited pathological features characteristic of thalassemia and the genetic analysis also confirmed this diagnosis. Two mutations in the ß-globin gene were detected.

The various genetic approaches described above were undertaken together to obtain exhaustive information concerning a specimen from the site of Tel Beit Shean, Israel (Mazar et al. 2002). The Middle Bronze Age skeleton of an 8-year-old child was subject to DNA analyses of descent, sex, and the presence of *M. tuberculosis*. The haplogroup of the specimen was determined as W, which occurs today in North African and some Middle Eastern populations. This result fit the hypothesis that the child was of Egyptian descent. A genetic test identified the individual as being male. The presence of *M. tuberculosis* DNA in conjunction with the absence of any skeletal lesions suggests that the individual suffered from miliary TB, which most probably was a cause of death. Due to the richness of the burial, this individual is referred to as an Egyptian prince.

Animal aDNA

Ancient DNA can also be a useful tool in archaeozoological analyses. It is used to confirm the results of morphological species identification. Moreover, it can reveal historical migrations and the distribution of animal populations. For example, aDNA studies can help to understand the process of domestication by identifying wild progenitors, the location and number of domestication events, or even the spread of early domesticates (see: Zeder et al. 2006 and Bruford et al. 2003 for a review). Methods of studying animal population genetics are similar to those for studying humans. MtDNA and Y-chromosomal analyses are routinely used to determine the level of genetic variation in a population and to construct phylogenetic trees. The Near East has always been considered as a place of domestication of several livestock species such as cattle and pig. Therefore, plenty of genetic analyses of both contemporary and ancient specimens from this region have been carried out; in particular for cattle, pigs, sheep, and goats.

Cattle (*Bos taurus*) domestication

Taurine cattle (*Bos taurus*) is a domesticated form of extinct wild ox (*Bos primigenius*). An analysis of nearly 400 cattle, belonging to 34 breeds from Europe, the Near East, and North Africa revealed that the mtDNA of *Bos taurus* is divided into four haplotypes T, T1, T2, and T3 (Troy et al. 2001). The European breeds belong almost exclusively to T3, while the African belong to the T1 haplotype. Near Eastern breeds harbor the largest portion of variation with all four haplotypes present (Troy et al. 2001). This led researchers to the conclusion that European cattle were domesticated in the Near East and were later introduced in Europe. Moreover, the phylogenetic trees of cattle in each region are star-shaped, which suggests a recent population expansion (Slatkin & Hudson 1991), most likely associated with domestication. Confirmation of these results came from the analysis of Neolithic and Bronze Age cattle remains from mainland Europe and the Near East. MtDNA sequences obtained in these studies resemble the modern ones. They also form star-like phylogenies mainly of the T3 haplotype (Edwards et al. 2004; Bollongino et al. 2006). Based on these sequences, popula-

tion expansion time was calculated at about 2000 (the Neolithic population) and 3000 (the Bronze Age population) years ahead of the age of the specimens from which the sequences were obtained, which again fits with initial domestication dates of 9–10,000 years ago (Bollongino et al. 2006). The single domestication centre hypothesis was confirmed by the results of the mtDNA sequences of wild ox (*Bos primigenius*). Nearly all the European wild ox possessed the P type of mtDNA haplotypes, distinct from the cattle's T type (Edwards et al. 2007). Additionally, the T type of mtDNA was found in one wild ox from Syria (Edwards et al. 2007). The picture of cattle domestication in the Near East and later spread of domesticates to Europe was complicated by the results of the Y chromosome analysis. Cattle from Europe exhibit two distinct Y chromosome haplotypes (Y1 and Y2) while Anatolian breeds display only one (Y2) (Götherström et al. 2005). Analysis of the aDNA of European wild ox revealed that they predominantly belong to the Y1 haplotype. The presence of a haplotype specific for European wild ox in the modern European gene pool suggests an introgression of European wild ox genes into the domestic cattle gene pool (Götherström et al. 2005).

Pig (*Sus scrofa*) domestication

A large scale genetic analysis of pig specimens from the Near East and Europe was performed by Larson et al. (2007). 543 mtDNA sequences were analyzed: 42 from modern and museum specimens, 280 previously published GenBank entries, and 221 sequences from ancient specimens from different sites in western Eurasia. This study revealed that the first domesticated pigs in Europe originated from the Near East and were introduced to Europe by humans. Wild boar haplotypes from European specimens dated to 11,000–5500 BCE (prior to pig domestication in this region) are distinct from the Near Eastern haplotypes (either modern or ancient, except for Armenian, see further). Wild European specimens dating to 5500–3900 BCE also have haplotypes specific for Europe. However, specimens described as domestic forms—from Germany (4 specimens from Eilsleben) and Romania (11 specimens from four sites) and two out of eight French specimens (from Bercy)—happen to have a Near Eastern (Y1) haplotype. All domestic pigs, dated to 3900–700 BCE, have European haplotypes which suggests that newly domesticated endogenous animals gradually replaced the descendants of the introduced ones. These data support the hypothesis that following the introduction of the domestic pig from the Near East, Europeans succeeded in domesticating the local wild boar.

The portion of the Larson et al. (2007) study, which focused on Armenian specimens, has also shown that the domestication of pigs was not unidirectional. The authors analyzed specimens dated to the 2nd millennium BCE and found that all shared the Near Eastern haplotype while specimens dated to the 7th century BCE – 13th century CE had a European haplotype. The authors suggest that it was probably the result of human migration or trade and exchange expansion during the later Iron Age.

Goat (*Capra aegagrus*)

Ancient DNA analyses in dealing with the subject of goat and sheep domestication are mainly carried out to support to the morphometric identification of these similar species. Examination of mitochondrial sequences of 9500 year-old Neolithic goats from the site of Abu Gosh,

Israel (Kahila Bar-Gal et al. 2002) made it possible to distinguish between bezoar goats (*Capra aegagrus*) and the Nubian ibex (*Capra ibex nubiana*). They could not be discriminated morphometrically because no ibex horns were found; ibex horns are the most reliable diagnostic characteristic of *C. ibex nubiana.*

The processes of domestication were obviously very complex and we are far from obtaining a complete description of the consecutive events that formed husbandry as we know it today. Genetic studies will not answer every question, but they provide a new and significant contribution to our knowledge of the subject.

Concluding remarks

New approaches and methods are still under development in the field of ancient DNA studies. Thanks to pyrosequencing (Margulies et al. 2005), whole mitochondrial genomes of ancient humans (Gilbert et al. 2008) and animals (Gilbert et al. 2007) have been sequenced. We know nearly the complete sequence of mammoth DNA (Miller et al. 2008). Ten years ago such a possibility was unimaginable. Recently, through molecular engineering, new DNA polymerases that are able to bypass sequence mismatches and lesions normally blocking Taq polymerases have been developed, which resulted in a 2-fold increase of PCR yields (d'Abbadie et al. 2007). The isolation of DNA enclosed in intergrown crystal aggregates of bone shows that relatively well-preserved DNA can be retrieved from specimens where standard procedures have failed (Salomon et al. 2005). These examples clearly indicate that progress in ancient DNA research continues to increase at a rapid rate. Ancient DNA analyses are therefore worth taking into consideration while planning new archaeological research.

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